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The undersigned hereby certify that they have read and recommend to the School of Graduate Studies for acceptance, a thesis entitled "The Effect of Ingested Chlortetracycline on the Activity of Some Hydrolases and on Some Organs Associated with the Digestive Process in Young Growing Swine" submitted by Ir. (Agric.) Marinus Adrianus Arnoldus Vonk, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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THE EFFECT OF INGESTED CHLORTETRACYCLINE ON THE ACTIVITY OF SOME HYDROLASES AND ON SOME ORGANS ASSOCIATED WITH THE DIGESTION PROCESS IN YOUNG GROWING SWINE

ABSTRACT

The hypothesis that part of the growth-promoting effect of antibiotics in rations for growing swine may be the result of a favorable influence of these drugs on protein and carbohydrate digestion in the intestinal tract was investigated. Sixteen pairs of weanling pigs were used; one pig of each pair was fed a practical growing ration, the other, this basal ration supplemented with chlortetracycline. Four pre-slaughter treatments involving differences in method of feeding and interval between last feed and slaughter were employed. After two to eight weeks on test the animals were killed by pairs and the weights of the pancreas, duodenal mucosa, and empty small intestine, as well as hydrolase activities of the pancreas and of the contents of the small intestine and of the cecum were determined.

The investigation necessitated the development of assay methods suitable for rapid and accurate determination of protease, amylase and cellulase activity in homogenates prepared from material collected from the experimental

animals. Turbidimetric, colorimetric and viscosimetric methods, devised for protease, amylase and cellulase determinations respectively, involved the derivation of regression equations with the aid of which enzyme activities of the homogenates could be calculated in terms of activities of equivalent weights of standard enzyme preparations.

The weight of the pancreas, and the protease and amylase content of this gland, were increased as a result of the feeding of the antibiotic. The effect of ingested chlortetracycline on pancreatic amylase was greater than that on rate of gain, weight of pancreas, or protease content of the gland. The fat content of the pancreas gland was not affected by dietary chlortetracycline.

The data obtained indicated that the feeding of chlortetracycline resulted in higher levels of protease, amylase and cellulase in the contents of the small intestine and cecum. The effect on amylase was greater than that on protease. The mean wet weight of the empty small intestine and the mean dry weight of the duodenal mucosa were lower for the pigs fed chlortetracycline but the differences were not significant.

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THE EFFECT OF INGESTED CHLORTETRACYCLINE ON THE ACTIVITY OF SOME HYDROLASES AND ON SOME ORGANS ASSOCIATED WITH THE DIGESTION PROCESS IN YOUNG GROWING SWINE

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REVIEW OF LITERATURE

Since the first reports appeared in 1949 and 1950 indicating that chlortetracycline present in *Streptomyces aureofaciens* fermentation residues induced improved rate of growth and efficiency of feed utilization when added to rations of chicks (93, 94) and pigs (37, 61), a great number of papers have been published which confirmed these results and showed similar effects from other antibiotics. Reviews of the literature by Braude and coworkers (20), Jukes (60), Jukes and Williams (62) and Maddock and Brackett (68) may be mentioned as excellent discussions of the several aspects in this field.

Mode of action of antibiotics

While the effect of dietary antibiotics on growth rate and efficiency of feed utilization of farm animals reared under normal conditions has been established, the mode of action of these drugs is, as yet, not completely elucidated. However, the bulk of the evidence

obtained to date supports the hypothesis that dietary antibiotics exert their growth-promoting effect by eliminating or suppressing subclinical infections. The following arguments, supporting the assumption that some microorganism or microorganisms in the intestinal tract must be considered as intermediate in the action of dietary antibiotics on the animal were forwarded by Jukes (60) and by Jukes and Williams (62). Since several antibiotics, effective as growth-promoting agents, are compounds without a common chemical property and exert their antibacterial action by different mechanisms, it would be difficult to understand their similar direct effect upon animal metabolism. Furthermore, animals reared under highly sanitary conditions or "germ-free" animals generally do not show a growth response when antibiotics are ingested and the efficiency of feed utilization as well as some physiological and biochemical characteristics of such animals are comparable to those of animals reared under less sanitary conditions but fed rations containing antibiotics. Injected antibiotics induce an improved growth rate only when they are excreted through the intestines via the bile like chlortetracycline and penicillin but not when they are excreted in the urine like bacitracin. On the other hand, antibiotics like bacitracin and streptomycin which do not readily enter the bloodstream when orally administered

exert a growth-promoting effect when supplemented in the ration. However, when orally administered antibiotics are easily absorbed into the bloodstream, infections at sites other than the intestines may be involved in the growth promoting effect (68).

Stunted animals usually show a striking response to ingested antibiotics; such animals may suffer from infections which inhibit normal growth (20). The feeding of antibiotics (for instance chlortetracycline at levels of 10 - 90 gm. per ton of feed) is a valuable tool in combating such diseases as gastro-enteritis and swine dysentry (68). These facts offer strong support to the hypothesis that antibiotics exert their growth-promoting action by suppressing infections.

With respect to the cause of the infections which supposedly are suppressed by dietary antibiotics, Jukes and Williams (62) suggested the possibility of a hitherto unknown organism while Maddock and Brackett (68) indicated that probably any form of infection which can be counteracted by antibiotics may be involved.

Catron (28) introduced the term "disease level" which he defined as the degree of contamination of the feed lot. According to this worker, the growth-promoting effect of antibiotics depends on the disease level of the environment. The disease level itself may vary from conditions under which perfectly healthy animals could be

reared, in which case no response to dietary antibiotics is observed, to conditions under which clinical symptoms or death would result, in which case ingested antibiotics show a striking effect on rate of growth or percentage survival of the animals. Since the disease level usually is uncontrolled in experiments, this concept may possibly explain the lack of agreement in magnitude of growth response to dietary antibiotics when results of different experiments are compared (20).

While, in general, evidence supports the hypothesis discussed above, contradictory observations also have been made indicating that other factors than the suppression of subclinical infections may play a role with respect to the mode of action of dietary antibiotics. Luckey (44) reported experiments in which oxytetracycline stimulated growth of chicks, and penicillin growth of poults, even when they were reared under "germ-free" conditions. He suggested that, apart from the suppression of subclinical infections, dietary antibiotics may have a direct effect upon the animals. He proposed the term "hormoligosis" for the latter effect, and suggested that any drug, while harmful when given in large quantities, may have a stimulating effect when given in proper doses.

Much work has been done to investigate the effect of ingested antibiotics on the intestinal microflora

(60, 62). However, the reports are confusing and only a suppression of clostridia appears to be a congruent result of experiments (62). With respect to some other kinds of common intestinal microorganisms (Coliforms, Lactobacilli, Aerobes, Anaerobes and Enterococci) an increase in number was observed in some experiments and a decrease in other experiments (17, 34, 35, 60, 69, 83, 87). On the other hand, Larson and Hill (65) working with hysterectomized pigs found no change in numbers of Aerobes, Anaerobes, E. Coli, Enterococci or Lactobacilli due to dietary antibiotics.

With regard to a vitamin-sparing action of ingested antibiotics the available evidence fails to supply conclusive information. While in some experiments the results indicate a sparing action, in others it appeared that the effect of ingested antibiotics was independent of vitamins (20, 60). In early work it was thought that such a sparing action might exist and that possibly it could be explained by the effect of dietary antibiotics on the intestinal microflora, either by suppressing organisms competing with the host for nutrients or by stimulating the multiplication of vitamin-synthesizing organisms. However, since an improved utilization of factors not synthesized by intestinal microorganisms has been reported, it would appear that, if a sparing action

exists, other factors also play a role. Burgess and coworkers (23) and Almquist and Maurer (1) found an increased level of vitamin A in the liver of chicks fed antibiotics as compared to that in control animals reared on the non-supplemented ration. Bowland (13) observed that the feeding of antibiotics to swine induced higher vitamin A levels in the liver. Ross and Yacowitz (84), Migicovsky and coworkers (74) and Bogdonoff and Shaffner (11) observed an improved utilization of calcium, while Pepper and coworkers (78) reported a better utilization of manganese by chicks when antibiotics were ingested. These observations were tentatively explained on the basis of the hypothesis that ingested antibiotics bring about a thinner and healthier intestinal wall, thus promoting absorption of nutrients (1, 32).

Effect of antibiotics on feed consumption

An increased daily feed consumption is generally observed when antibiotics are ingested. Bowland and McElroy (16), in a review of experiments with swine conducted at the University of Alberta, reported an average increase of 18% in the daily feed consumption when chlortetracycline was fed until the pigs reached an average weight of 75 pounds. The average daily feed consumption was increased by 7% in experiments in which the antibiotic was fed to 125 pounds. Braude and coworkers (20) reviewed

reports, dealing with the effect of ingested antibiotics on rate of gain and feed efficiency of swine, from various experimental stations in North America. In the experiments reviewed, an average increase in daily feed consumption of 23% was observed when chlortetracycline was ingested.

Increased rate of gain due to ingested chlortetracycline appeared to be mainly the result of increased daily feed consumption in experiments with chicks reported by Scott and coworkers (86) and similar conclusions were arrived at by Brown and coworkers (21) from experiments with swine. However, in experiments with chicks and poults, in which the feed intake of the chlortetracycline- or penicillin-fed animals was restricted to that consumed by the controls, the antibiotics still induced improved rate of gain and feed efficiency (39).

Relationship between efficiency of feed utilization and rate of gain

If increased daily feed consumption causes an increased rate of gain it may induce *ipso facto* an improved efficiency of feed utilization by growing animals. Since the higher rate of gain will reduce the feeding period necessary to reach a specific body weight, a smaller proportion of the feed will be used to meet maintenance requirements. The ratio between amount of feed used for production and for maintenance thus becomes more favorable

the shorter the feeding period. In a genetic study with Duroc swine Dickerson and Grimes (40) found a highly significant correlation between daily gain and pounds of feed required per pound gain ($r = -0.66$). Therefore, an improved efficiency of feed utilization may result from, (a) higher retention coefficient of nutrients and (b) increased daily retention of nutrients or increased rate of gain.

Effect of antibiotics on water retention

An apparent improvement of feed efficiency resulting from the feeding of antibiotics would be obtained if these drugs increased the amount of water retained to a larger extent than the retention of solid nutrients. Water retention is generally not recorded in experiments and a relatively greater amount of water retained would decrease the amount of feed necessary per pound of gain. However, if this were the case, the moisture content of the carcass would be increased when antibiotics are ingested but no significant difference in moisture content of pork carcasses resulting from the feeding of antibiotics was observed in experiments reported by Pierce (79) and by Huang and McCay (57).

Effect of antibiotics on nitrogen retention

Rats

In a study with rations containing cottonseed oil

meal or soybean oil meal as protein supplements, reported by Berry and Schuck (6), chlortetracycline stimulated the daily feed intake and induced a greater rate of gain. The daily nitrogen retention was increased when chlortetracycline was supplemented in a cottonseed oil meal ration containing 18 or 21% of protein but the nitrogen retention coefficient was reduced approximately 4% due to the feeding of the antibiotic. On a soybean oil meal ration containing 15% of protein, increased daily feed intake, rate of gain, nitrogen retention and a small increase in the nitrogen retention coefficient (63.6 versus 62.2%) was observed when the antibiotic was ingested. When this ration was supplemented with an amino acid mixture containing methionine, lysine, and phenylalanine the retention coefficient for nitrogen was 71.3% in the presence and 71.0% in the absence of the antibiotic.

An increased daily retention of nitrogen and a reduced retention coefficient resulting from ingested oxytetracycline were observed by Hartsook and Johnson (49) in experiments with rats on a methionine deficient ration. When the ration was supplemented with methionine both daily nitrogen retention and nitrogen retention coefficient were significantly greater when the antibiotic was ingested.

In experiments with rats on an all vegetable protein

ration fortified with vitamin B_{12}, reported by Black and Bratzler (10), ingested streptomycin slightly increased daily feed intake and nitrogen retention but reduced the nitrogen retention coefficient. Similar results were reported by Knoebel and Black (64) when rats were fed a low protein ration.

Swine

No significant increase in rate of gain or nitrogen retention was observed, when chlortetracycline was added to rations for pigs, under conditions of equalized feed intake (22). With pigs fed *ad libitum* significantly greater daily gain and significantly improved efficiency of feed utilization were observed when the antibiotic was ingested but no retention data were reported in this case. Braude and Johnson (19) reported experiments in which neither feed intake nor nitrogen retention was increased significantly by dietary chlortetracycline, while rate of gain and efficiency of feed utilization showed a significant improvement.

In experiments conducted by Russo and coworkers, reported by Jukes (60), ingested chlortetracycline improved the nitrogen retention coefficients in young growing swine fed a ration containing 12.5% protein. The mean value for the nitrogen retention coefficients was 28.6% for the control animals and 39.5% for the antibiotic-fed pigs.

The available evidence indicates that, while nitrogen retention usually was increased when antibiotics were ingested, retention coefficients sometimes were reduced. Hohls (53) offered a possible explanation for the decrease in the nitrogen retention coefficient when antibiotics are fed; dietary antibiotics may increase daily feed consumption to levels at which more protein is absorbed than can be used for protein anabolism. The maximum protein anabolism will be reached sooner the higher the biological value of the protein. Therefore, according to this hypothesis, one would expect that the effect of ingested antibiotics on nitrogen retention coefficients would be more deleterious the higher the biological value of the protein in the ration. However, in the experiments reported by Hartsook and Johnson (49), ingested oxytetracycline affected the nitrogen retention coefficient unfavorably when rats were on a methionine deficient ration and favorably when this amino acid was supplemented in an otherwise identical ration.

Effect of antibiotics on energy retention

Rats

Mean daily fat retention was increased by 8% when streptomycin was added to a vitamin B_{12} fortified all vegetable protein ration in experiments reported by Black and Bratzler (10). Hartsook and Johnson (49)

reported similar results in experiments with rats on rations either deficient in methionine or fortified with this amino acid. The energy retention coefficient was increased from 44.1% to 60.1% for the animals on the methionine deficient ration and from 46.4% to 48.9% on the ration fortified with this amino acid when oxytetracycline was fed. The differences in the energy retention coefficient were statistically significant in both cases. Forbes (46) reported that dietary streptomycin or chloramphenicol did not affect the basal energy metabolism of rats on a soybean oil meal ration either deficient in methionine or supplemented with the amino acid.

Swine

No direct evidence appears to be available with respect to the effect of antibiotics on energy retention by swine. Braude and Johnson (19) reported that the urinary water output, expressed as a percentage of the water intake, was considerably greater for swine receiving dietary chlortetracycline than for the control animals. They suggested that the ingested antibiotic might have decreased the metabolic activity of the experimental animals which could cause a smaller loss of water by respiration and sweating. From this one might conclude that the ingested antibiotic may have decreased the loss

of energy as heat and consequently increased the energy retention.

The available evidence indicates that the effect of ingested antibiotics on fat retention is more favorable than that on nitrogen retention (10, 49), however, such evidence is insufficient as yet.

Effect of antibiotics on protein requirements

Chicks

West and Hill (101) pointed out that an improved efficiency of protein utilization does not *per se* indicate a protein-sparing action of the dietary antibiotic, since gross feed efficiency and protein efficiency are mathematically related for any one level of protein in the ration:

$$\frac{\%\ \text{Protein}}{100} \times \text{Gross feed efficiency} = \underset{\text{(lb. protein per lb. gain)}}{\text{protein efficiency}}$$

Therefore, only when the protein efficiency is improved to a greater extent than the feed efficiency, based on this equation, would a true lowering of protein requirements be indicated.

Comparison of the optimum protein levels of rations fortified with antibiotics and those of rations not so fortified has been used to reach a conclusion regarding the effect of these growth-promoting agents on protein requirements of the animal. Experiments with turkey starter rations reported by Slinger and coworkers (90)

indicated that a protein content of 26% in the ration was too low when no penicillin was added but that optimum feed efficiency was obtained at this protein level when the antibiotic was present. Maximum growth rate and optimum feed efficiency at an 18% protein level in broiler rations supplemented with antibiotics and at a 20% protein level in rations without antibiotic were reported by West and Hill (101). These results were not confirmed by Slinger and coworkers (88, 89) for broilers or by Scott and coworkers (86), Matterson and coworkers (70) and Biely and coworkers (7) for chicks.

Swine

Catron and coworkers (29) studied the interaction of the effect of protein levels and of chlortetracycline supplementation on rate of growth and feed efficiency of swine on a corn-soybean oil meal ration. The rations fed up to a bodyweight of 75 lb. contained either 20, 16 or 14% protein; at 75 lb. and again at 150 lb. of body weight the protein content of each ration was lowered by 3 percentage points. Each ration was fed to 2 groups of animals, one group receiving the dietary antibiotic and the other acting as control group. When the ration did not contain antibiotic the growth rate of the animals on the 20, 17, 14% or on the 16, 13, 10% protein ration was not significantly different, but the rate of gain of the

pigs on the 14, 11, 8% protein ration was significantly lower. With respect to the rations containing chlortetracycline the daily gain of the animals on the 14, 11, 8% ration was not significantly lower than on the rations with higher protein levels. The growth rate obtained with the rations to which the antibiotic had been added was considerably higher than that obtained with the rations not containing chlortetracycline. Results regarding efficiency of feed utilization showed the same trend. Bowland and McElroy (15), Hoefer and coworkers (52) and Jensen and coworkers (59) reported similar results. However, Wahlstrom (98) found that dietary penicillin did not affect the optimum protein level of the ration and Hironaka and Bowland (51) obtained a greater response to ingested penicillin on a 17% than on a 15% or 13% protein ration.

The experimental evidence discussed above indicates that, at least under some conditions, optimum protein levels are lower when rations are supplemented with antibiotics. This poses a semantic problem as to the meaning of the term "protein-sparing" action of dietary antibiotics. Criteria for a real protein-sparing action of antibiotics as listed by Jensen and coworkers (59) are quoted below.

"1. Pigs would have to show a greater response to

antibiotic feeding at lower than at higher protein levels. There is some, but insufficient, evidence of this to date.

2. There would have to be a statistic interaction between protein level and antibiotics if the latter actually affect the response to protein levels. Analysis of the data accumulated at the Iowa Station fails to show an interaction.
3. Antibiotic feeding would have to improve nitrogen retention at any protein level, especially at low levels."

Meade (72) applying these criteria did not find a protein-sparing effect of antibiotics in his experiments with swine. The authors forwarding these criteria observed that the optimum protein level was lower when chlortetracycline was fed to pigs but when the first two criteria were applied no protein-sparing action appeared to exist. With respect to the third criterion it may be suggested that, rather than an increased nitrogen retention, an improvement beyond that observed for energy retention would have to be obtained before a real protein-sparing action exists. The relation between nitrogen and energy retention as influenced by dietary antibiotics was discussed above under the heading "Effect of antibiotics on energy retention" and it would appear that the effect

of dietary antibiotics on energy retention is greater than that on nitrogen retention.

Effect of antibiotics on fat content of swine carcasses

Several reports of experiments to investigate the effect of ingested antibiotics on carcass quality have appeared in the literature. A small increase in the fat content of the carcass resulting from the feeding of antibiotics has been reported (3, 14, 16, 79, 98, 99, 102). On the other hand, Catron and coworkers (29) observed a slight decrease in the fat content of the carcass when chlortetracycline was fed. While none of the tested differences was statistically significant, the evidence suggests that dietary antibiotics may induce a greater fat content of the carcass.

The possibility that antibiotics have a more favorable effect on energy retention than on nitrogen retention as discussed under the heading "Effect of antibiotics on energy retention" is supported by the data with respect to the effect of antibiotics on the fat content of pork carcasses. Therefore, it would appear that dietary antibiotics, apart from increasing rate of gain and improving the feed efficiency, influence the relationship between energy and nitrogen metabolism, which in turn may affect the optimum ratio carbohydrate:protein in the ration.

Effect of antibiotics on the absorption of nutrients

Retention of nutrients is the final outcome of a series of processes involving digestion, absorption, metabolism and excretion. Absorption as commonly measured in digestion trials depends on the efficiency of enzymatic digestion and absorption of nutrients. Presently the problem of absorption as influenced by dietary antibiotics will be discussed without making a distinction between absorption *sensu stricto* and digestion so that the results from digestion trials are relevant.

Rats

Carrol and coworkers (27) reported an improved digestion coefficient for protein associated with the feeding of chlortetracycline in experiments with rats fed a ration containing heated soybean oil meal as protein supplement. When raw soybean oil meal was used as protein supplement the protein digestion coefficient was increased but the difference was not significant. Similar results were obtained by Berry and Schuck (6) and Forbes (46).

Swine

Huang and McCay (57) fed a corn-soybean oil meal ration to swine and observed an increased rate of gain when oxytetracycline was ingested at a level of 33 mg. per kg. of feed. The effect of the dietary antibiotic

on digestion of nutrients was measured in the fifth and the fourteenth weeks of the experiment. Increased digestion coefficients due to the feeding of the antibiotics were observed for dry matter, protein, carbohydrate, fat and minerals in the fifth week. The ingested antibiotic improved the digestion coefficients for these nutrients also during the fourteenth week with the exception of carbohydrate for which a slightly lower digestion coefficient was observed. Brown and coworkers (22) working with pigs under conditions of equalized feed intake reported a non-significant increase in digestion coefficients for protein and energy when chlortetracycline was ingested. Burnside and coworkers (25) reported experiments in which the protein digestion coefficient was significantly increased when chlortetracycline was added to a medium protein ration or when both vitamin B_{12} and chlortetracycline were added to a low or medium protein ration. The supplementation of both vitamin B_{12} and chlortetracycline to a low protein ration resulted in a significantly higher digestion coefficient for the ration as a whole. The feeding of the antibiotic induced a greater daily feed consumption without reducing the digestion coefficients for protein. Increased protein digestion coefficients due to the feeding of chlortetracycline at a level of 10 or 20 gm.

per ton of feed were observed by Russo and coworkers as reported by Jukes (60).

Effect of antibiotics on the intestinal wall

No direct evidence appears to be available with respect to a possible effect of ingested antibiotics on the actual absorption of digested nutrients. However, data have been reported which indicate that such an effect may exist. Coates (31) reported that the weight of chick intestines was lower when the ration was supplemented with penicillin as compared to that of chicks fed a ration to which no antibiotic had been added. Coates and coworkers (32) reported that penicillin, arsanilic acid and, to a lesser extent, chloramphenicol induced a lower gut weight and gut length when fed to chicks. Since the decrease of the weight was greater than that of the length, the authors concluded that the intestinal wall was thinner when the chicks were fed the supplemented ration, Histological examination of the gut wall failed to reveal any difference between that of the penicillin-fed and of the control chicks; no difference in the fat or moisture content of the gut wall resulting from the ingested penicillin was noted. Penicillin fed to chicks reared in isolation units had no effect on rate of gain or weight and length of intestines. The conclusion which these authors reached,

based on the greater decrease in the weight than in the length of the intestines, has been questioned. This phenomenon does not necessarily involve a thinner gut wall since it could be caused by a decrease in the diameter of the intestinal lumen (2).

Sieburth and coworkers (87) reported that the mesenteric blood vessels appeared to be more prominent in chicks fed rations supplemented with penicillin or chlortetracycline as compared to these blood vessels in control chicks fed the basal ration. The authors suggested that either a greater absorption or a decreased production of vasoconstricting amines in the intestines of the antibiotic-fed chicks may have been responsible for this phenomenon.

Enzymatic hydrolysis of nutrients in the intestinal tract

The review of the literature on the foregoing pages brought forward evidence which indicates that dietary antibiotics induce an improved absorption and retention of nutrients. The effect on absorption coefficients appears to be favorable but the available evidence is not conclusive. The effect of dietary antibiotics on enzymatic hydrolysis of nutrients has not been investigated to the author's knowledge. Yet the available evidence with respect to absorption and retention suggests that such a study might contribute to a more complete

understanding of the effect of dietary antibiotics on rate of growth and efficiency of feed utilization. A greater retention of nutrients requires a greater availability of same to the animal and a greater absorption requires more nutrients to be digested. Consequently the enzymatic hydrolysis of nutrients occupies a key position in the utilization of feed.

The most important enzymes acting at the first stages of digestion of nutrients were chosen by considering the proximate composition of common feeds for growing swine and their normal digestibility. Disregarding vitamins and minerals a ration consisting of 15 parts of soybean oil meal and 85 parts of barley may be considered as approximately normal for young growing swine in Western Canada. The composition of such a ration would be (76):

Water	10%
Protein	16%
Fat	3%
Fiber	6%
N-free extract	61%
Minerals	4%

For most rations the nitrogen-free extract is almost exclusively starch, which thus appears to be the most important constituent quantitatively, followed by

protein and fiber. The digestion coefficient for protein is usually approximately 75-80%, for starch 85-90% and for fiber 20% (36) indicating that fiber is of minor importance from the point of view of nutrition. However, evidence exists that undigested fiber affects the digestion of protein unfavorably (67, 75); the significance of fiber in the ration is thus greater than would appear from its availability as such.

The first stages of protein digestion to polypeptides in weanling mammals are brought about by pepsin in the gastric juice and trypsin and chymotrypsin secreted by the pancreas into the duodenum (92a). The optimum pH of pepsin is approximately 2 (47b) and since the hydrogen ion concentration in the intestine of swine is close to neutral (42) it is unlikely that pepsin is of major importance in the digestion of proteins in intestinal material. Pancreatic juice is secreted by the pancreas into the duodenum when the stomach contents are passed into the small intestine . Hydrochloric acid present in the stomach contents causes the liberation of secretin from the intestinal mucosa into the bloodstream. Secretin affects the volume of the pancreatic juice secreted but its enzyme content is regulated by pancreozymin, liberated from the intestinal mucosa (42). Direct nervous action possibly also influences the enzyme content of pancreatic

juice (48). Trypsin is secreted as trypsinogen and activated by enterokinase present in the *succus enericus* or by trypsin itself once it is produced. Chymotrypsin is secreted as chymotrypsinogen and activated by trypsin. The duodenal area of the small intestines contains two types of glands: Brunner's glands and Lieberkühn's glands. It is impossible to distinguish between the contribution of these two types of glands towards the *succus entericus* (45).

Pancreatic amylase plays an important role in the digestion of starch in the intestines of swine. The intestinal mucosa also contributes amylase but the amount of this is negligible as compared to the amount present in the pancreatic juice (45). Pancreatic amylase is an α-amylase hydrolysing 1,4 α-glycosidic linkages in the interior of the amylose or amylopectic molecule.

Cellulose present in plant fiber, though not hydrolysed by mammalian enzymes (92b) is digested to some extent by the action of cellulolytic microorganisms in the intestines (63). Cellulose represents a linear array of D-glucopyrannose units linked by 1,4 β-glycosidic linkages which are hydrolysed by cellulases, cellobiose being the final degradation product of this process (47a).

PRELIMINARY STUDIES CONDUCTED TO DEVELOP AND TEST HYDROLASE ASSAY METHODS

Preliminary experiments conducted to develop methods to be employed in the measurement of hydrolase activity of material derived from experimental animals are described in this section. The first experiments were conducted with "standard" enzyme preparations of known activity. Assay methods were developed to measure the activity of these enzyme preparations using regression equations. Appropriate methods to prepare enzyme extracts from the animal material to be studied were devised and the hydrolase assay methods were, when necessary, modified to permit the determination of enzyme potency in these extracts. The methods, as finally employed, together with the results obtained in the investigation are presented in a later section in the form of three papers to be submitted for publication.

Part A. Experiments with "standard" enzyme preparations

Digestion mixture

All assays were done with the following mixture:

8 ml. of substrate solution

1 ml. of enzyme solution

1 ml. of distilled water.

The pH at which the digestion mixture was to be

maintained during digestion presented some difficulties. The optimum pH for trypsin *in vitro* is approximately 8, of swine pancreatic amylase 5.5 - 6.0 (42), and of cellulase, dependent on the origin, 3.0 - 7.0, however, it would appear that the optimum pH for cellulases most likely to be active in swine intestines is approximately 4.0 - 6.0 (80); these values indicate that these hydrolases would not act at their optimum pH as found *in vitro* in the intestines of swine where the hydrogen ion concentration is approximately at neutral. However, since conditions *in vitro* differ from those *in vivo*, the optimum pH under the latter conditions may differ from that determined in *in vitro* assays. Vonk (97) reported an optimum pH of 7 for trypsin activity *in vitro* when bile salts were added to the digestion mixture. In view of these considerations it was decided to maintain the pH in the digestion mixture at 7.0 to approach the hydrogen ion concentration existing in the intestines of swine (42).

The substrate solution was maintained at pH 7.0 with phosphate buffer. The concentration of the phosphate buffer was 0.2 M in the substrate solutions for protease and amylase assays and 0.02 M in the substrate solution for cellulase assays; the reason for the lower concentration in the latter case will be discussed later.

Protease

The assay for protease activity was based on the determination of the concentration of undigested casein in the hydrolysate obtained by digesting a 0.1% casein solution with a protease preparation. A determination of the turbidity, after precipitation of the undigested casein in the hydrolysate with 5 ml. of a 4% acetic acid solution added to 10 ml. of the hydrolysate, was carried out with an Evelyn photoelectric colorimeter using a 620 $m\mu$ filter (30, 82). The amount of acetic acid to be added was determined by conducting trials with a 0.1% casein solution buffered at pH 7.0 with a 0.2 M phosphate buffer. These trials indicated that 5 ml. of a 4% acetic acid solution would be a suitable addition to precipitate the undigested protein.

The accuracy of this method to determine protease activity depends primarily on the accuracy with which casein concentrations may be estimated. In order to determine this, a series of casein solutions with different concentrations varying from 0.0 to 1.0 mg. per ml. was prepared and the photometric density, after precipitation of the casein as described above, was determined. During these experiments it was noted that temperature affected the photometric density. It was therefore decided to conduct these experiments in the

same way as the actual protease assays were to be done. The solutions were placed in assay tubes, which were shaken at 37°C. for 2 hours after which they were placed in a constant temperature waterbath at 25°C. for 30 minutes. The acetic acid was then added and the photometric density was determined. The results of these experiments conducted on different days are reported in Table I.

Table I

Photometric Densities of Casein Solutions at Different Concentrations After Precipitation of the Protein with Acetic Acid

Conc. casein (mg./ml.)	Photometric density*
0.0	0.000
0.2	0.122 ± 0.0010
0.4	0.248 ± 0.0015
0.6	0.364 ± 0.0027
0.8	0.472 ± 0.0018

* All values are means of 8 determinations.

When solutions were made up and treated as described above and 1 ml. of distilled water was replaced by 1 ml. of a 50 p.p.m. chlortetracycline solution no significant differences in photometric densities attributable to the

presence of the antibiotic were observed. It was therefore concluded that chlortetracycline per se at the concentration used had no effect on the photometric density.

From the standard errors reported in Table I it was concluded that the concentration of casein could be determined with a high degree of accuracy by the method described above. The use of a 0.1% casein solution as substrate solution appeared to be suitable and this concentration was used in further determinations. Since 8 ml. of substrate solution were present in 10 ml. of digestion mixture the casein concentration in the digestion mixture was 0.8 mg. per milliliter.

The most suitable range of concentrations of "enzyme" mixture (3 parts Pancreatin 3 X USP to 1 part Intestinal Hog Mucosa (30)) was determined by conducting digestion trials with a series of concentrations of the enzyme preparation. Photometric densities, useful for the calculation of regression equations relating photometric density to "enzyme" concentration, were obtained with concentrations of the enzyme preparation up to 0.02 mg. per ml. of digestion mixture. The regression equations with the aid of which the protease potency of unknown enzyme solutions was determined are reported in Paper I.

Amylase

A number of methods of assay for amylase activity based on the determination of substrate concentration, color with iodine, viscosity, degree of hydrolysis and degradation products have been developed (77). The most suitable of these methods for the present purpose appeared to be the colorimetric determination of the change in color with iodine. When starch is hydrolysed it passes through stages of erythrodextrins and achrodextrins until the final product maltose appears. A color change from blue via purple to brown occurs when iodine is added to hydrolysates at these varying stages of digestion. This fact complicates the choice of filter to use for colorimetric determinations. Filters varying from 390 to 680 mμ have been used to study starch and starch degradation (5, 8, 9, 12, 56, 71). Since the purpose of the present experiments was to establish a regression equation, which necessarily involved colorimetric determinations over a wide range of degrees of digestion, the choice of the filter to use was of necessity a compromise. The filter selected was determined by measuring the photometric density of a starch-iodine complex produced by adding 1 ml. of a 0.002 N iodine solution to 10 ml. of a 0.01% starch solution. Maximum photometric density was observed with a 660 mμ filter and this filter was

thus chosen.

In Table II the photometric densities and standard errors of different concentrations of starch solutions after the addition of 1 ml. of a 0.002 N. iodine solution to 10 ml. of the starch solutions are reported. During these experiments it was observed that the accuracy of the determinations could be increased considerably by storing aliquots of the iodine solution at 5°C. in separate glass stoppered flasks each of which contained a sufficient amount for 1 series of tests.

Table II

Photometric Densities of Starch-Iodine Complexes at Different Concentrations of Starch

Conc. starch (mg./ml.)	Photometric density*
0.00	0.0000
0.02	0.2709 ± 0.0033
0.04	0.5540 ± 0.0014
0.06	0.8190 ± 0.0011
0.08	1.0800 ± 0.0010
0.10	1.3470 ± 0.0008

* All data are means of 8 determinations.

The results of experiments conducted to determine the

effect of chlorteracycline on the photometric density of starch-iodine complexes are shown in Table III. It appeared that the presence of chlortetracycline at a concentration of 5 p.p.m. decreased the photometric density of starch-iodine complexes when the concentration of starch was low. Another important feature of the photometric densities of starch-iodine complexes becomes evident when the data given in Table III are compared to those of Table II. It will be noted that for equal concentrations of starch the photometric density reported in Table III is lower than the corresponding one in Table II. The reason for this discrepancy lies in the fact that while the starch solutions used for the experiments reported in Table II had been stored for 20 hours at 5°C., those used in the experiments which are reported in Table III had been stored for some days at the same temperature. After this observation starch solutions were always stored for approximately 20 hours at 5°C. before use.

Table III

The Influence of Chlortetracycline at a Concentration of 5 p.p.m. on the Photometric Density of Starch-Iodine Complexes

Conc. starch (mg./ml.)	Photometric density Control	Photometric density Chlortetracycline
0.02	0.201	0.160
0.04	0.446	0.429
0.06	0.643	0.650
0.08	0.892	0.903

Experiments were conducted to examine whether this direct effect of the antibiotic on the photometric density of the starch-iodine complexes could be avoided. Starch solutions varying from 0.2 to 2.0% containing 5 p.p.m. of chlortetracycline were prepared together with solutions of the same concentrations but without the antibiotic. The solutions were diluted 1/200 (final concentration of chlortetracycline 0.025 p.p.m.) after which 1 ml. of the iodine solution was added and the photometric density determined. The results of these experiments as reported in Table IV indicate that no direct effect of chlortetracycline on the photometric density of the starch-iodine complexes existed when the antibiotic was present at a concentration of 0.025 parts per million. Therefore, if the digestion assays were done with a 2% starch solution and the hydrolysate were diluted 1:200 after digestion, the digestion mixture could contain 5 p.p.m. of chlortetracycline, while the concentration in the hydrolysate after dilution would be 0.025 p.p.m. at which level the antibiotic would not affect the photometric density of the starch-iodine complexes. However, the possibility was considered that, though the antibiotic at the lower concentration no longer affected the photometric density of starch-iodine complexes, it still might influence the photometric density of the iodine complexes of starch

fission products in the hydrolysate. The following experiment was therefore conducted. Digestion trials were done with 8 ml. of a 2% starch solution and 1 ml. of Pancreatin 3 X USP solutions at suitable concentrations. After a 2 hour digestion period 1 ml. of water was added to one duplicate and 1 ml. of a 50 p.p.m. chlortetracycline solution to the other duplicate. The hydrolysates were then diluted 1:200, the iodine solution added and the photometric density determined. The results of this experiment are given in Table V and indicate that, within the range of usable colorimeter readings, chlortetracycline at a concentration of 0.025 p.p.m. did not affect the photometric density of iodine complexes of starch fission products.

Table IV

The Effect of Chlortetracycline at a Concentration of 0.025 p.p.m. on the Photometric Densities of Starch-Iodine Complexes

Conc. starch (mg./ml.)	Photometric density* Control	Photometric density* Chlortetracycline
0.00	0.000	0.000
0.01	0.128	0.129
0.03	0.409	0.410
0.05	0.683	0.680
0.07	0.949	0.944
0.09	1.222	1.222

* All data are means of 4 determinations.

Table V

The Effect of Chlortetracycline (0.025 p.p.m.) on the Photometric Density of Iodine Complexes of Starch Fission Products

Conc. pancreatin (mg./ml.)	Photometric density* Control	Photometric density* Chlortetracycline
0.000	1.126	1.140
0.004	0.453	0.449
0.006	0.111	0.120
0.008	0.042	0.037
0.010	0.007	0.014

* All data are means of 4 determinations.

With the aid of the information gained in the preliminary trials the method of amylase assay as given in Paper I was devised.

Cellulase

Water soluble carboxymethyl cellulose (CMC) is a suitable substrate for cellulase assays (55, 81, 95, 96). This cellulose derivative is soluble in water but insoluble in organic solvents. Several methods were attempted to arrive at a rapid and accurate method of cellulase assay. A turbidimetric determination of CMC concentration after precipitation with organic solvents failed as the nature of the precipitate was unsuitable for turbidimetric measurements. The determination of

reducing sugars either by the method of Jansen and MacDonnel (58) or by the micro-determination of the copper number (41) was not sufficiently accurate for the present purpose.

The intrinsic viscosity of suspensions of high polymers may be expressed by the following equation:

$$(\eta) = k M^{a} \qquad (38)$$

where (η) is the viscosity at infinitesimal concentration, M the molecular weight, and k and a constants. When the molecule is fairly straight as in the case of cellulose, and probably of carboxymethyl cellulose, the value of a is approximately 2, indicating that the viscosity is proportional to the square of the molecular weight. Hydrolysation of the CMC molecule thus causes a decrease in the viscosity of the CMC suspension. This property was used by Holden and Tracey (55) to develop a qualitative viscosimetric method for the detection of cellulase activity, using CMC as substrate.

In the present work a quantitative cellulase assay method was devised, based on the determination of the relative viscosity of CMC hydrolysates. A 2% CMC suspension in 0.2 M phosphate buffer at pH 7.0 was prepared and the relative viscosity of different dilutions of this suspension with the phosphate buffer was determined using Ostwald viscosimeters. The results of this experiment are shown

in Table VI. It was found that the concentration of the CMC suspension had to be very high in order to ensure a workable range of relative viscosities. In fact, when the concentration of CMC was high enough to obtain a relative viscosity of 2.00, no regular flow through Ostwald viscosimeters occurred.

Table VI

Relative Viscosities of Different Concentrations of CMC Suspensions in 0.2 M. Phosphate Buffer

Conc. CMC %	Relative viscosity
0.0	1.00
0.1	1.11
0.2	1.22
0.3	1.34
0.4	1.49
0.5	1.75
0.6	1.92
0.7	- *
0.8	-
0.9	-

* No regular flow through viscosimeters.

Carboxymethyl cellulose forms a lyophilic suspension in water and one of the properties of lyophilic suspensions was used to overcome the above mentioned difficulty. The relative viscosity of lyophilic suspensions depends on their ionic strength. Therefore the 2% CMC suspension was diluted with distilled water,

in order to dilute simultaneously the carboxymethyl cellulose and the phosphate buffer which was the most important factor with respect to the ionic strength of the suspension. The relative viscosities of the suspensions thus obtained were determined and the results of this experiment are reported in Table VII.

From the data shown in Table VII it may be concluded that a wide range of relative viscosities may be ensured by diminishing the ionic strength of the suspension without unduly raising the concentration of carboxymethyl cellulose. It was decided to use provisionally a 0.3% CMC suspension in 0.03 M phosphate buffer for the following preliminary experiments.

Table VII

Relative Viscosities of CMC Suspensions of Different Concentrations and Ionic Strengths

Conc. CMC %	Molarity of phosphate buffer	Relative viscosity
0.1	0.01	2.07
0.2	0.02	2.32
0.3	0.03	2.63
0.4	0.04	2.81
0.5	0.05	2.81

Since the cellulase activity in the intestines of

weanling pigs was assumed to be quite small, it was decided to use a long digestion period (18 hours) in order to be able to detect small differences in the hydrolase activity.

The direct effect of chlortetracycline on the relative viscosity of suspensions of CMC was determined in the same manner as described under the heading "amylase". Digestion mixtures containing 8 ml. of the CMC suspension used (0.3% in 0.03 M phosphate buffer) and 1 ml. of enzyme 19 solutions of arbitrary concentrations prepared as described in Paper I were shaken for 18 hrs. at 37°C., after which 1 ml. of distilled water was added to one duplicate and 1 ml. of a 50 p.p.m. chlortetracycline solution to the other duplicates. The relative viscosities of the hydrolysates thus treated are reported in Table VIII.

Table VIII

The Direct Effect of Chlortetracycline (5 p.p.m.) on Relative Viscosities of Enzyme Digests of CMC Suspensions

Conc. Enzyme 19 (mg./ml.)	Relative viscosities Control	Relative viscosities Chlortetracycline
0.000	2.64	2.65
0.002	1.85	1.84
0.004	1.68	1.67
0.006	1.53	1.53
0.008	1.49	1.49
0.010	1.46	1.46

The data indicate that at a level of 5 p.p.m. chlortetracycline had no effect on the relative viscosities of CMC or its degradation products.

When the regression equation relating relative viscosity of the hydrolysate to cellulase activity, as reported in Paper I, was worked out it became apparent in the experiments conducted for this purpose that greater accuracy could be ensured by decreasing the concentration of CMC in the suspension to 0.2% combined with a decrease in the concentration of the phosphate buffer to 0.02 molar. In order to profit from this increased accuracy the concentration of carboxymethyl cellulose and phosphate buffer was altered accordingly. This change did not affect the results obtained in the preliminary trials as reported above.

Part B. Experiments with enzyme extracts of unknown potency

The material with which these preliminary experiments were conducted was obtained from a packing plant. While this material was derived from swine weighing approximately 200 lbs. and the methods were to be applied to weanling pigs, it was hoped that the principles of the methods could be devised using material from the heavier animals. Only minor adjustments, involving homogenization period and dilution of homogenates to determine their hydrolase activities, were made for assays on material from the experimental animals used in the actual investigation.

Enzyme extracts

Enzyme extracts were prepared by homogenization of pancreas glands, duodenal mucosae, and contents of small intestines and of ceca, since it was decided to determine the hydrolase activity of this material from the experimental animals. The homogenates were prepared in a room kept at 5°C. using electric blenders.

Pancreatic material

The pancreas was cleaned, weighed and cut into small pieces after which approximately 2/3 of the material was stored for approximately 18 hours. This sample was then placed in an electric blender with approximately 5 times its weight of chilled distilled water and homogenized.

Initially it was thought that 2 periods of 5 minutes each would be sufficient for complete homogenization, however, it was found that small flakes of tissue still remained intact and a further 5 minute period was required to complete the process. Using another type of blender it was then noted that homogenization for 5 minutes sometimes caused the temperature of the material to rise to more than 30°C. which was considered undesirable. Two minute periods were therefore introduced, the total time of homogenization being 15 minutes, which was later reduced to 10 - 15 minutes with material from the weanling animals.

It proved to be necessary to centrifuge the homogenates for 45 minutes at 1500 r.p.m. in order to obtain a satisfactory separation of the solid material. The liquid was then collected and the process was repeated with the precipitate after which the two aliquots of liquid were mixed and stored as described in Paper II.

Duodenal mucosa

Although the whole of the intestinal wall is lined with glandular tissue, the duodenal glands which may contribute essential material towards the *succus enterinus* (45) only occur in the first 3 - 5 m. after the pylorus in swine (85). It was therefore decided that only the anterior 3 m. of the small intestines should be used to prepare homogenates of intestinal mucosa. Two systems were used; the first consisted of cutting the intestines in small pieces, while the second involved everting the intestine and scraping the mucosa off with the edge of a slide. Prior to both these treatments the intestinal contents had been removed by gently squeezing them out after which the intestines were washed by running water through them. The mucosa was carefully blotted with a paper towel after the intestine had been drained for approximately 2 hours. It was found that the scrapings gave more satisfactory results than did the cut material as will be shown below so that only scrapings were used

in later experiments.

Satisfactory homogenization could be ensured following the same method as described for pancreatic material. The amounts of water used were twice those used for pancreas homogenates for reasons to be discussed below.

Contents of small intestine and cecum

Homogenization of contents of small intestine and cecum was done in the same manner as described for pancreatic material using half the amount of water in order to increase the hydrolase activity per ml. of homogenate.

Measuring enzyme activity

Protease

The activity of protease was estimated by colorimeter readings after digestion of casein. To investigate a possible influence of enzyme extracts *per se* on readings obtained, the following experiment was carried out:

To 8 ml. of the casein solution 1 ml. of water and 1 ml. of enzyme extract was added. After a two hours digestion period the enzyme activity could be calculated from the colorimeter readings using the regression equations reported in Paper I. Combined with these, others were carried out in which 8 ml. of water replaced the 8 ml.

of casein solution which should give readings of zero since no undigested casein could be present in the hydrolysate and again others in which 2 ml. of water were added to the casein solution so that no digestion could occur. Furthermore trials were carried out by adding the enzyme extract immediately before the readings were taken, thus without a digestion period. The results obtained with these trials are reported in Table IX, indicating that the enzyme extract *per se* did not affect the colorimeter readings when the photometric density was at maximum (the no digestion column) nor when it was at minimum (the no substrate column), taking normal errors into account.

Table IX

The Influence of Enzyme Extracts *per se* on Photometric Densities as Applied in Protease Assay

Extract	Dilution	Photometric density: After digestion	No digestion	No substrate
Pancreas-Int. mucosa	10^{-4}	0.323	0.469	0.0033
Contents of small intest.	10^{-2}	0.093	0.465	0.0022
Cecal contents	10^{-2}	0.405	0.465	0.0011
Water	-	-	0.469	0.0000

The pancreatic homogenate exhibits proteolytic activity only after it has been mixed with the homogenate from intestinal mucosa which contains the essential enterokinase. The problem arose as to which of the two homogenates, derived from the intestinal mucosa, would cause the greatest activity and in what proportion such homogenate should be combined with that of the pancreas. In order to find an answer to these problems the following experiment was conducted.

The extract of 0.12×10^{-4} gm. pancreas was mixed with several levels of the two intestinal homogenates. At any particular level of intestinal mucosa the weights derived from the scraping and from the cut material were equal. Digestion trials were conducted utilizing these enzyme preparations. Table X gives the results obtained in this experiment. It will be seen that, except at very low levels, the homogenate derived from scrapings caused greater enzyme activity than did that derived from the cut material. It was therefore decided to use homogenates derived from scrapings.

The data reported in Table X indicate that a sharp rise in proteolytic activity occurred up to a point where the concentration of intestinal mucosa was 0.4 times that of the pancreas. It was assumed that this rise was due to enterokinase action and that the slow increase in enzyme activity after this point was caused

by the presence of pancreatic or other proteolytic agents in the intestinal mucosa homogenate. The proportion in which the homogenates of pancreas and intestinal mucosa should be mixed would therefore appear to be 0.4 parts of intestinal mucosa to 1 part of pancreas expressed on a wet weight basis. In order to have a slight excess of enterokinase it was decided to combine the two in such a manner that the proportion would be 0.5 gm. intestinal mucosa to 1 gm. of pancreas.

By making the volume of the pancreatic homogenate up to 10 times the weight of the homogenized material and the intestinal mucosa to 20 times its original weight it was possible to obtain suitable mixtures by combining equal volumes of the two extracts.

Table X

The Protease Activity of a Definite Concentration of Pancreas Homogenate when combined with Two Types of Intestinal Mucosa Homogenates in Different Proportions

gm. Intest. mucosa: gm. Pancreas	Protease activity*	
	Scraping	Cut material
0.0	0.00	0.00
0.2	5.97	6.56
0.4	11.45	7.99
0.6	12.64	7.99
0.8	13.42	8.35

* Protease activity expressed in micrograms enzyme mixture described in Paper I.

Amylase

The method used to determine amylase activity was similar to that used for protease activity, both involving measurement of photometric densities. In this case also a mixture of equal volumes of pancreas and intestinal mucosa homogenates was used.

It has been reported that duodenal glands secrete amylase (42), though the amount secreted is probably negligible as compared to that present in pancreatic juice (45). The effect of the presence of intestinal mucosa homogenate in the pancreatic enzyme extract was investigated by conducting the following experiment.

The amylase activities per gm. pancreas and per gm. intestinal mucosa were determined using separate homogenates. Combined with this, the amylase activity per gm. pancreas was determined using a mixture of the two homogenates so that the amylase activity of the pancreas material and of the material derived from the intestinal mucosa could be compared and also a possible effect of material from the intestinal mucosa on pancreatic amylase could be detected. Since it was considered possible that dietary chlortetracycline might affect the relationship between amylase activities of the two tissues, these experiments were conducted with material from the first two pairs of experimental pigs in order to determine a possible effect of the ingested drug. The results of these experiments

are given in Table XI. The amylase activity of the intestinal mucosa was negligible as compared to the activity of the pancreatic material and the presence of the homogenate from the intestinal mucosa did not affect the amylase activity of the pancreatic homogenate since this activity per gm. pancreas was of the same magnitude whether the determination was done in the presence or in the absence of the intestinal mucosa. Dietary chlortetracycline had no effect on the relationship between the amylase activity of the two tissues.

Table XI

The Amylase Activity per gm. of Intestinal Mucosa and of Pancreas and the Effect of Material Derived from the Intestinal Mucosa on the Activity of Pancreatic Amylase

Homogenate used	Amylase activity* Control	Amylase activity* Chlortetracycline fed
Pancreas-Intest. mucosa	471	706
Pancreas	474	705
Intestinal mucosa	0.54	0.62

* Amylase activity expressed in mg. Pancreatin 3X USP (Paper I).

The direct effect of the enzyme extracts on the photometric density which was employed to determine enzyme activity was determined in the same manner as

described for protease. The results of this experiment are reported in Table XII, indicating that, when normal errors are taken into account, the enzyme extracts per se had no effect on the photometric density.

Table XII

The Effect of Enzyme Extracts per se on Photometric Densities Applied in Amylase Assays

Extract	Dilution	Photometric density		
		After digestion	No digestion	No substrate
Pancreas-Int. mucosa	10^{-4}	0.0011	1.046	0.0011
Contents of small intest.	10^{-3}	0.274	1.059	0.0011
Cecal contents	10^{-2}	0.903	1.046	0.0000
Water	-	-	1.046	0.0000

Cellulase

The activity of cellulase was measured viscosimetrically. To determine the direct influence of enzyme extracts the following experiments were conducted.

Using several dilutions of an extract of Enzyme 19 a simple procedure was followed. To each of two tubes 8 ml. of a carboxymethyl cellulose solution and 1 ml. aliquots of Enzyme 19 extract were added. The contents were digested for 18 hrs. Immediately prior to measuring the viscosity 1 ml. of homogenate was added to one tube

Table XIII

The Direct Influence of Enzyme Extracts on the Viscosity at Several Stages of Digestion of Carboxymethyl Cellulose Compared to the Influence of the Same Extract on the Viscosity of Water

Contents 9 ml.	Addition 1 ml.	Rel. Viscos.	Rel. visc. (water): Rel. visc. (enzyme extract)
Water	water	1.00	
	P-I*	1.07	0.93
Digested	water	2.41	
	P-I	2.31	1.04
CMC	water	2.02	
	P-I	2.08	0.97
	water	1.97	
	P-I	1.90	1.04
	water	1.85	
	P-I	1.92	0.96
Water	water	1.00	
	CC**	1.09	0.92
Digested	water	2.26	
	CC	2.46	0.92
CMC	water	2.02	
	CC	2.18	0.93
	water	2.04	
	CC	2.21	0.92
	water	1.97	
	CC	2.13	0.92
Water	water	1.01	
	Sic***	1.05	0.95
Digested	water	2.32	
	Sic	2.44	0.95
CMC	water	2.04	
	Sic	2.11	0.97
	water	2.11	
	Sic	2.24	0.94
	water	1.95	
	Sic	2.07	0.94

* P-I: Pancreas - Intestinal mucosa homogenate.

** CC: Cecal content homogenate.

*** Sic: Homogenate of contents of small intestines.

and 1 ml. of water to the other. The viscosity of water and of a mixture containing 9 ml. of water and 1 ml. of enzyme extract was also measured. The results of these experiments are reported in Table XIII.

The data shown in Table XIII show that the enzyme extracts affected the relative viscosity when they were present in water and also when they were present in digested carboxymethyl cellulose. The last column of the table indicates that the ratio $\frac{\text{Rel. Visc. (water)}}{\text{Rel. Visc. (enzyme extract)}}$ was constant as far as the extracts derived from cecal contents and the contents of the small intestines were concerned. Therefore, a correction factor, representing this ratio, could be introduced to correct for the presence of the enzyme extract. This correction factor could be determined by experiments with water containing the particular enzyme extract. The possibility that shaking for 18 hours at 37°C. may affect the relative viscosity of a mixture of 1 ml. of intestinal or cecal homogenate and 9 ml. of water was investigated by measuring the relative viscosity of these mixtures before and after shaking. Since the relative viscosity was not changed by the treatment it was concluded that the correction factor, mentioned above, could be determined with fresh material.

Such a correction factor could not be determined in the case of the Pancreas - Intestinal mucosa homogenate

due to variability of the influence of the homogenate upon the viscosity. Consequently, any quantitative estimation of cellulase activity in this homogenate was impossible by the technics used. However, it was most likely that this homogenate would not possess any cellulase activity at all and a qualitative method was considered to be suitable. Such a method did not present great difficulties. The comparison of the relative viscosity of a carboxymethyl cellulose suspension to which the homogenate had been added before a digestion period, with the viscosity of the same suspension to which the homogenate had been added after the digestion period, was suitable for this purpose.

Loss of enzyme activity during storage

Homogenates were stored under toluene at approximately 1 1/2°C., and the loss of enzyme activity during the storage was determined by measuring the activity per ml. of homogenate on different days. Unfortunately it was impossible to determine this activity immediately after preparation of the homogenate. The activity found 1 day after preparation was taken as 100% and the activities observed on other days were expressed in percentage of that of the first day. The results of these experiments are shown in Table XIV indicating that a loss of enzyme activity occurred in the following order: Cellulase >

Amylase > Protease. In the experiments during the actual investigation this order of loss in activity was taken into account by timing the activity tests accordingly so that cellulase activity was tested first followed by amylase and protease.

Table XIV

Changes in Enzyme Activity during Storage

Days after preparation	Homogenate	Enzyme activity per ml. of homogenate*					
		Protease		Amylase		Cellulase	
		mg.	%	mg.	%	mg.	%
1	Pancreas-Intest. mucosa	26.1	100	20.6	100	-	-
2		25.9	99	20.0	97	0	-
4		25.1	96	18.3	89	0	-
6		22.2	85	17.7	86	0	-
8		20.1	77	15.8	77	0	-
1	Small intestinal content	29.15	100	3.59	100	-	
2		29.65	102	3.49	97	0.0024	100
4		27.80	95	3.26	91	0.0021	88
6		24.40	84	3.12	88	0.0008	33
8		22.00	75	2.69	75	0.0001	0.4
1	Cecal content	0.549	100	0.0148	100	-	
2		0.555	101	0.0153	103	0.0517	100
4		0.486	89	0.0139	94	0.0310	60
6		0.496	90	0.0111	75	0.0100	19
8		0.504	91	0.0103	70	0.0050	9.5

* Enzyme activities are expressed in mg. of enzyme preparations described in Paper I.

Calculations of enzyme activity

Enzyme activities were determined with appropriate dilutions of the homogenates taking care that the readings

obtained after digestion permitted the use of the regression equations reported in Paper I. The following calculations were then carried out.

$$\frac{\text{Enzyme activity in digestion mixture}}{\text{Dilution}} = \text{Enzyme activity per ml. of homogenate}$$

$$\frac{\text{Enzyme activity per ml. of homogenate}}{\text{gm. material per ml. of homogenate}} = \text{Enzyme activity per gm. material}$$

$$\text{Enzyme activity per gm. material X weight of material} = \text{Total enzyme activity}$$

$$\frac{\text{Enzyme activity per gm. material}}{\text{\% dry matter}} \text{X } 100 = \text{Enzyme activity per gm. dry matter.}$$

PAPER I - METHODS FOR THE DETERMINATION OF PROTEASE, AMYLASE AND CELLULASE ACTIVITY

Abstract

Methods for the determination of protease, amylase and cellulase activity of unknown solutions permitting accurate measurements over a wide range of enzyme concentration in a single determination are described. Regression equations were obtained relating: (a) protease concentrations to the optical densities of casein hydrolysates precipitated with an acetic acid solution ($r = -0.9806$), (b) optical densities of starch-iodine complexes formed in starch hydrolysates to amylase concentrations ($R = 0.9918$), and (c) cellulase concentrations to relative viscosities of carboxymethyl cellulose hydrolysates ($r = -0.9554$). The influence of chlortetracycline on the activity of these hydrolases *in vitro* was investigated and no effect was detected.

Introduction

The effect of antibiotic supplements on rate of growth and efficiency of feed utilization suggests that they may cause improved digestion and absorption of nutrients. Experiments of Burnside and coworkers (25) indicate an ability to digest more feed without a decrease in digestion coefficients resulting from supplementation of swine rations with chlortetracycline,

while increased absorption of digested material by chicks receiving chlortetracycline or penicillin supplement was suggested by Sieburth and coworkers (87).

No evidence is available to the author's knowledge regarding possible effects of ingested antibiotics on the activity of hydrolases associated with the digestive process in growing swine. In order to study the possibility that such effects exist it was essential to devise rapid and accurate methods for the determination of the activity of typical hydrolases involved in the first stages of digestion. These methods are described in the first part of this paper. In the second part evidence is presented to show that, at low concentration, chlortetracycline in the assay tubes does not affect the accuracy of results obtained.

A. Determination of Protease, Amylase and Cellulase Activity

Digestion - Digestion was done in Evelyn colorimeter tubes or in 20 x 150 mm. test tubes with a mixture of Pancreatin 3 X USP* and Intestinal Hog Mucosa** as a source of protease; Pancreatin 3 X USP as a source of amylase, and Enzyme 19*** as a source of cellulase.

* National Biochemical Corporation, Cleveland, Ohio.

** Wilson Laboratories, Chicago, Illinois.

*** Rohm and Haas Company, Philadelphia, Pennsylvania.

Each tube contained: 8 ml. of substrate solution, 1 ml. of enzyme solution and 1 ml. of distilled water. The digestion mixture was maintained at pH 7.0 in order to approach that of the intestinal tract of pigs (42).

Protease Assay - The method of assay for protease activity was adapted from that of Riesen and coworkers (82) in which protease activity is determined by measuring the concentration of undigested casein in partially hydrolysed digests. A 3% stock solution of casein was prepared by a modification of the method given by Hawk and Bergeim (50). Thirty gm. of casein were stirred with 70 ml. of 0.45 N sodium hydroxide and 800 ml. of water. When the casein was in solution the volume was made to 1000 ml. with distilled water. This stock solution was unstable during storage at 5°C. and as a consequence new solutions were prepared when the reading obtained on a diluted blank after the addition of acetic acid indicated the necessity. Before use, the stock solution was diluted to 0.1% casein with 0.2 M phosphate buffer at pH 7.0. The final pH was adjusted to 7.0 with a few drops of 8 N sodium hydroxide.

A mixture of Pancreatin and Intestinal Hog Mucosa was prepared immediately prior to use by the method of Clandinin (30) in which 3 parts of Pancreatin 3 X USP and 1 part of Intestinal Hog Mucosa are suspended in distilled water, filtered and brought to concentrations equivalent to 0.02 to 0.2 mg. of dry enzyme mixture per milliliter.

The digestion mixture was shaken in calibrated colorimeter tubes in an incubator room at 37°C. for 2 hours and cooled in a constant temperature waterbath at 25°C. for 30 minutes. Five ml. of a 4% acetic acid solution were added to precipitate the undigested casein after which the turbidity was measured immediately by means of an Evelyn photoelectric colorimeter using a 620mμ filter.

Amylase Assay - The photometric density of the blue starch-iodine complex has been employed by several workers including Bourne and coworkers (12) and Bird and Hopkins (8) in studies of starch degradation. Based on this procedure a method for the measurement of amylase activity was devised. A 2% starch substrate solution was prepared by stirring 10 gm. of soluble starch* with 100 ml. of distilled water and pouring the solution into 375 ml. of boiling phosphate buffer at pH 7.0. The solution was boiled for 1 minute and stirred while cooling to room temperature; 1.2 gm. of sodium chloride was added and the solution, after dilution to a final volume of 500 ml., was stored at 5°C. for use 18 to 24 hours after preparation. An enzyme solution containing 0.1 mg. of Pancreatin per ml. was prepared immediately before use and 1 ml. portions of appropriate dilutions were added to assay tubes to give final Pancreatin concentrations as shown in Table XV.

* Reagent grade, Merck and Company, Inc., Rahway, New Jersey.

The assay tubes were shaken for 2 hours in an incubator room at $37^{\circ} \pm 0.1^{\circ}C$. Somogyi (91) reporting a method for the determination of amylase activity in blood samples, advised digestion periods of 30 minutes or less. However, preliminary trials indicated that, with the present method, smaller differences in activity of enzyme solutions could be detected when a 2-hour digestion period was used as compared to a 30-minute period. Following the hydrolysis period the samples were diluted 1:200 with distilled water and 10 ml. aliquots were transferred to calibrated colorimeter tubes. One ml. of a 0.002 N iodine solution was added and the photometric density of the starch-iodine complex was determined with an Evelyn photoelectric colorimeter using a 660 mμ filter. The iodine solution was prepared by dissolving 12.7 gm. of iodine in 25 ml. of 10 M potassium iodide solution (43), making to 500 ml. and diluting 1:100 with distilled water. The final solution was stored in the dark at $5^{\circ}C.$, for periods not exceeding 4 weeks, in separate glass-stoppered flasks each of which contained an amount sufficient for one series of determinations. The stability of the iodine solution was checked at each assay by conducting a blank determination.

Cellulase Assay - Since cellulose is insoluble in water, it is not a suitable substrate for measuring cellulase

action especially when the activity is of a low order. Several water soluble derivatives of cellulose have been studied in order to determine their suitability as substrates (55, 81). Results of these studies indicate that the activity of very small quantities of cellulase may be detected by viscosimetric methods when carboxymethyl cellulose (CMC)* is used as substrate (55).

A 2% CMC stock solution was prepared at pH 7.0 in 0.2 M phosphate buffer and stored at 5°C. The substrate solution was obtained by diluting the stock solution 1:10 with distilled water. The cellulase solution was prepared by stirring 5 gm. of Enzyme 19 in 95 ml. of distilled water for 20 minutes and centrifuging the suspension at 1500 r.p.m. for 30 minutes (54). The supernatant liquid was collected, made to 100 ml. and stored under chloroform at 5°C. Under these conditions the cellulase activity remained constant from the second to the fifth week of storage. Further dilution with distilled water was made to concentrations in the digestion tubes equivalent to 0.0001 - 0.0004 mg. of dry Enzyme 19 per milliliter.

The assay tubes were shaken for 18 hours at 37°C. after which they were held in boiling water for 30 minutes and cooled in a constant temperature waterbath at 25°C.

* CMC 50, Hercules Powder Company, Hopewell, Virginia.

for one hour. The relative viscosity of the hydrolysates was then determined in a waterbath at 25°C. using Ostwald viscosimeters with flow time of 85 - 103 seconds for 5 ml. of distilled water.

Accuracy of Assays and Method of Calculating Activity of Enzyme Preparations of Unknown Potency

The accuracy of the methods was tested by conducting assays on different days using a series of tubes in duplicate to which enzyme preparations of known equivalent of dry enzyme had been added. Enzyme equivalents per tube, together with mean assay readings on the digests and standard deviations for individual readings are listed in Table XV for protease and amylase and in Table XVI for cellulase. Regression equations, relating readings of photometric density or relative viscosity to enzyme concentrations, were derived from the raw data and used in the estimation of the activity of unknown enzyme solutions. Straight line regression equations were found to be most accurate for protease and cellulase determinations whereas a quadratic equation considerably improved the accuracy of the method for amylase estimation. A table relating known concentrations of Pancreatin to observed photometric densities was computed from the quadratic equation and employed in estimating the potency of amylase preparations of unknown concentration.

The calculated regression equations were:

Protease - $P = 0.01945 - 0.04477\ D$
$(r = -0.9807;\ D.F.\ 70)$

where P is the concentration of Pancreatin-Intestinal Hog Mucosa mixture in mg. per ml. and D the photometric density;

Amylase - $D = 1.17491 - 228.272\ A + 10920\ A^2$
$(R = 0.9918;\ D.F.\ 62)$

where D is the photometric density and A is the concentration of Pancreatin in mg. per ml.;

Cellulase - $C = 0.002247 - 0.000963\ V$
$(r = -0.9554;\ D.F.\ 34)$

where C is the concentration of Enzyme 19 in mg. per ml. and V the relative viscosity of the hydrolysate. This equation is accurate only for enzyme concentrations between 0.0000 and 0.0004 mg. per milliliter.

Table XV

Mean Photometric Densities of Casein and Starch Hydrolysates after Digestion with Varying Levels of Enzyme*

"Enzyme" concentration mg. /ml.	Photometric Densities	
	Casein hydrolysate	Starch hydrolysate
0.000	0.473 ± 0.0020	1.145 ± 0.0045
0.002	0.389 ± 0.0019	0.781 ± 0.0051
0.003	---	0.623 ± 0.0024
0.004	0.335 ± 0.0016	0.468 ± 0.0013
0.005	---	0.300 ± 0.0016
0.006	0.290 ± 0.0013	0.137 ± 0.0030
0.008	0.237 ± 0.0012	0.030 ± 0.0004
0.010	0.181 ± 0.0017	0.006 ± 0.0016
0.012	0.135 ± 0.0009	---
0.016	0.084 ± 0.0011	---
0.020	0.040 ± 0.0038	---

* All means are based on 4 determinations in duplicate.

Table XVI

Mean Relative Viscosities of Carboxymethyl Cellulose Hydrolysates after Digestion with Varying Levels of Enzyme 19

Number of determinations	"Enzyme" concentration mg./ml.	Relative viscosity
12	0.0000	2.33 ± 0.055
4	0.0001	2.14 ± 0.010
4	0.0002	2.04 ± 0.017
4	0.0003	1.96 ± 0.050
12	0.0004	1.93 ± 0.019

B. Effect of Chlortetracycline on the Hydrolases *in vitro*

Methods

The influence of chlortetracycline on the hydrolases was measured by conducting assays in the presence or absence of this antibiotic. From a review of the literature it appeared that the concentration of chlortetracycline in the small intestines is extremely variable. However, using data of Yacowitz and Bird (103) and Sieburth and coworkers (87), it was calculated that growth response with chicks has been obtained at a concentration of approximately 5 p.p.m.; therefore this concentration was employed in *in vitro* digestion.

In setting up the comparison 8 ml. of appropriate

substrate, 1 ml. of enzyme solution and 1 ml. of water were added to the control tubes while 8 ml. of appropriate substrate, 1 ml. of enzyme solution and 1 ml. of a 50 p.p.m. solution of chlortetracycline* were added to the treatment tubes. Digestion periods and enzyme concentrations employed will be found in Tables XVII and XVIII.

Table XVII

Influence of Chlortetracycline on the Activity of Protease and Amylase

Digestion period (hr.)	Mean Enzyme Activity[1]			
	Protease[2]		Amylase[3]	
	Control	Treatment[4]	Control	Treatment[4]
1/2	3.81	3.76	2.03	2.03
1	5.03	5.14	3.12	3.12
1 1/2	6.25	6.25	4.38	4.39
2	9.95	9.95	5.90	5.90
Enzyme added (γ/ml.)	10	10	6	6

1) All means are based on 8 determinations.
2) In micrograms protease mixture per ml.
3) In micrograms pancreatin per ml.
4) Chlortetracycline 5 p.p.m.

* Research Division, American Cyanamid Ltd., New York, N.Y. Aureomycin hydrochloride.

Table XVIII

Effect of Chlortetracycline on Cellulase Activity of Enzyme 19

Digestion period (hr.)	Mean Cellulase Activity[1) 2)] Control	Treatment [3)]
4 1/2	0.16	0.16
9	0.25	0.23
13 1/2	0.31	0.32
18	0.39	0.38
Enzyme added (γ /ml.)	0.40	0.40

1) All means based on 8 determinations.
2) In micrograms Enzyme 19 per ml.
3) Chlortetracycline 5 p.p.m.

An analysis of variance of the raw data summarized in Tables XVII and XVIII is presented in Table XIX. The analysis indicates that under the conditions employed chlortetracycline at a concentration of 5 p.p.m. had no effect on the activity of any of the hydrolases *in vitro*. In preliminary work it was found that at a level of 100 p.p.m. the antibiotic inhibited protease activity.

Table XIX

Analysis of Variance of the Effect of Chlortetracycline on the Activity of Hydrolases

Source of variation	F Values		
	Protease	Amylase	Cellulase
Treatment vs. control	0.20	1.52	2.28
Digestion period	6,737**	27,217**	109**
Interaction	0.06	0.20	1.36

** $P < 0.01$.

PAPER II - THE EFFECT OF DIETARY CHLORTETRACYCLINE ON THE DRY WEIGHT, FAT CONTENT, PROTEASE ACTIVITY AND AMYLASE ACTIVITY OF THE PANCREAS OF GROWING SWINE

Abstract

Results of experiments to investigate the effect of dietary chlortetracycline on rate of gain of growing pigs, efficiency of feed utilization, pancreas dry weight, plus crude fat content and hydrolase activity of the pancreas are presented. Chlortetracycline fed at a level of 20 grams per ton of feed caused an increase in rate of gain and efficiency of feed utilization by pigs between the ages of 9 and 17 weeks. An effect on the pancreas was indicated by a significantly greater dry weight, total hydrolase activity and hydrolase activity per gram dry matter of the glands from animals receiving the supplemented ration. The difference remained significant after adjustment for body weight by covariance in the case of amylase activity but not for dry weight or protease activity, indicating that the effect of chlortetracycline on amylase activity was more pronounced than that on rate of gain, pancreas dry weight or pancreatic protease activity. No significant difference in the crude fat content of the pancreas attributable to the inclusion of chlortetracycline in the ration was observed.

Introduction

Reports have appeared in the literature which indicate that ingested antibiotics may affect specific animal organs. Calesnick and coworkers (26) observed an increased thyroid weight and a lower I^{131} uptake of this gland per gram body weight when penicillin or chlortetracycline was administered to rats at the rate of 1 mg. per kg. of feed. These results were not confirmed by Libbye and Meites who also failed to observe an anti-thyroid effect when penicillin was supplemented in chick rations (66). Menge and Conner (73), working with chicks, noted an increased thyroid weight per pound of body weight when chlortetracycline or thiouracil was ingested; however, since thiouracil depressed growth while a growth-promoting action was indicated for the antibiotic, the authors suggested that the mechanism of action of chlortetracycline on the thyroid differed from that of thiouracil.

A greater dry weight of the liver per pound body weight was observed by Burgess and coworkers (24) in chicks fed a ration supplemented with penicillin, but Braude and coworkers (18), working with pigs weighing approximately 150 lb., found no significant differences attributable to dietary chlortetracycline for the weights of liver, kidneys and spleen, adjusted for body weight.

Cohen and Rachmilewitz (33) reported that dietary chlortetracycline stimulated growth of rats suffering from chronic alloxan diabetes and suggested that this was due to a specific effect of the drug on metabolism.

An excellent review of possible explanations for the effects of antibiotics on rate of growth and efficiency of feed utilization is included in a recent monograph by Jukes (60). The pancreas plays an important role in the digestive process in supplying hydrolases associated with the digestion of a variety of nutrients. The present study was undertaken to evaluate the possible effect of dietary chlortetracycline on the weight and digestive enzyme content of the pancreas in growing swine.

Experimental

Between June and December 1955, 14 Yorkshire and 6 crossbred Lacombe x Yorkshire, 8 to 9-week-old weanling pigs were paired on a littermate, sex and weight basis. An additional 12 purebred Yorkshire weanling pigs, paired on a sex and weight basis, were on experiment during February 1956. During the experimental period the pigs were housed in an old pig barn where a positive response to antibiotics had been obtained in previous experiments. Lot I received the small grain basal ration shown in Table XX, while Lot 2 was fed the same ration supplemented

with 20 gm. of chlortetracycline* per ton of feed. None of the pigs used had received supplementary antibiotic in their creepfeed rations nor was any antibiotic added to their dams' lactation ration.

Table XX

Basal Ration for Lot I

Ground barley	lb.	84
Soybean oil meal	lb.	10
Linseed oil meal	lb.	5
Ground limestone	lb.	0.5
Iodized salt	lb.	0.5
Vitamin A	I.U./lb.	500
Vitamin D	I.U./lb.	100
Vitamin B_{12}	mg./lb.	0.003

Four feeding and management procedures were used. These involved ad libitum feeding, paired hand-feeding, starvation for 16-24 hours before slaughter, and slaughter 2 hours after feed was last offered. A detailed outline of the feeding and management treatments employed is reserved for Paper III because differences in these treatments were of more significance in relation

* Research Division, American Cyanamid Ltd., New York, N.Y. Aureomycin hydrochloride.

to results obtained on intestinal and cecal material than to those secured on the pancreas. Liveweights were recorded weekly. Feed consumption was recorded by groups weekly for pigs fed ad libitum, and daily for those that were pair-fed.

At 2, 3, 4, 6 or 8 weeks after being placed on test the pigs were killed by pairs by severing the jugular vein. The pancreas and small intestines were removed immediately after slaughter and brought to a room kept at 5^{o}C., where the pancreas was freed from extraneous tissue and cut into small pieces. The anterior 3-meter section of the small intestine was cleaned, washed, everted, and blotted dry. The mucosa was then scraped off with a microscope slide. After removal of samples for dry matter determinations, the remaining pancreatic tissue and intestinal mucosa were stored at 5^{o}C.

After 16 to 20 hours of storage a large sample, approximately 2/3 of the pancreas, was homogenized in an electric blender with approximately 5 times its weight of chilled distilled water. The actual homogenization time was approximately 15 minutes, but in order to prevent an excessive rise in temperature during the process the homogenizer was switched off for 2-minute periods at 2-minute intervals. The homogenate was then centrifuged for 45 minutes at 1500 r.p.m. The supernatant liquid

was collected and the process was repeated with the precipitate. The two portions of supernatant liquid were mixed, and diluted to a volume at which 1 ml. of supernatant (hereinafter referred to as homogenate) corresponded to approximately 0.1 gm. of pancreas. The exact concentrations were calculated from the recorded weights and volumes. Homogenates of the intestinal mucosa were prepared in a similar manner except that the amount of water used was calculated to give a final tissue concentration one-half that of the homogenate derived from the pancreas of the same animal. Procedures described above were carried out in a room held at 5°C. The homogenates were stored under toluene at 1°C. for assay approximately 18 hours after preparation.

The results of preliminary assays showed that a mixture of equal volumes of pancreatic and intestinal mucosa homogenates gave: (a) maximum readings for protease activity, and (b) the same readings for amylase activity as equivalent dilutions of pancreatic homogenate alone. Consequently assays for protease, amylase and cellulase activity were performed on mixtures of equal volumes of pancreatic and intestinal mucosa homogenates from the first 10 pairs (7 in the case of cellulase) of experimental pigs. Assays were done by the methods described in Paper I and dilutions of the pancreas-

intestinal mucosa homogenates were used to give readings that permitted the application of the regression equations presented in Paper I to estimate the activity of the homogenates from the test pigs. Assays for cellulase were discontinued after no activity was detected in homogenates from the first 7 pairs of animals.

In preliminary trials known concentrations of Pancreatin 3 X USP and of Intestinal Hog Mucosa were added to one-half of pancreatic material and to one-half of intestinal mucosa scrapings respectively prior to homogenization, while the other half was homogenized without the addition of any enzyme. The concentrations of the added enzyme preparations were calculated to give a Pancreatin-Intestinal Hog Mucosa mixture of 3 parts to 1 part when the homogenates were prepared and mixed as described above. The results of assays conducted on mixtures of homogenates from pancreatic material and from intestinal mucosa scrapings to which no enzyme had been added and to which enzyme had been added indicated recoveries of 102% of added protease, and 96% of added amylase.

The pancreas glands from the last 6 pairs of pigs were used to determine whether any difference existed, with respect to fat content of this gland, between pigs fed the basal ration and those fed the basal ration

supplemented with chlortetracycline. After trimming in the usual manner the glands were dried and ground to permit accurate sampling. Fat determinations were done on 2-gram samples by the A.O.A.C. (4) method. Dry matter was determined by drying to a constant weight in a vacuum oven at 95° - 100°C.

Results

Rate of Gain and Efficiency of Feed Utilization

Data for mean daily gain and efficiency of feed utilization are summarized in Table XXI. Pigs fed chlortetracycline grew faster and required less feed per pound gain than their controls regardless of whether they were fed *ad libitum* or were limited in their feed intake to the amount eaten by their pair-mates. Results for the pigs fed *ad libitum* were not analysed separately, but when the rate of gain data for all 16 pairs were combined and analysed the differences between control and antibiotic-fed pair-mates were significant.

Table XXI

Effect of Dietary Chlortetracycline on Mean Daily Gains and Feed Required per Pound Gain

Lot Chlortetracycline	1 -	2 +	Between treatment F value	Number of Pairs
Fed by paired-feeding technic				
Daily gain . . .lb.	0.71	0.81	22.10	13
Feed/lb. gain. .lb.	3.39	3.04	14.22*	13
Group-fed ad libitum				
Daily gain . . .lb.	0.52	0.85	-	3
Feed/lb. gain. .lb.	3.24	2.76	-	3
All animals combined				
Daily gain . . .lb.	0.67	0.81	17.69*	16
Feed/lb. gain. .lb.	3.27	2.99	-	16

* $P < 0.01$.

Weight of Pancreas, Protease and Amylase Activity of Pancreas

Evidence will be presented in Paper III which indicates that differences in amount of feed eaten by control and supplemented pigs fed *ad libitum*, as well as the interval of time between the last feed and slaughter, are factors that complicate the interpretation of results for hydrolase assays conducted on intestinal and cecal contents. In an attempt to determine whether these factors influenced the effect of chlortetracycline on either pancreas weight or hydrolase activity of

pancreas-intestinal mucosa homogenates, F values were calculated for interaction of chlortetracycline supplementation with, (a) method of feeding, and (b) interval between last feed and slaughter. These values were all insignificant. Thus it was concluded that analysis of variance of results for all 16 pairs of pigs was valid for comparison of control and treatment pairs with respect to pancreas weight and hydrolase activity. All statistical analyses were done on a within pair basis to remove the high between pair variability in weight which existed due to the fact that pairs were slaughtered at intervals between 2 and 8 weeks after being placed on test.

Pair-mate pigs receiving a ration supplemented with chlortetracycline grew faster and were thus heavier at slaughter than their controls, and highly significant correlations existed between body weight and: (a) pancreas dry weight, $r = 0.91$, (b) total pancreatic protease activity, $r = 0.67$, and (c) total pancreatic amylase activity, $r = 0.60$. An analysis of covariance was done on the data for these factors in an attempt to adjust for within pair differences in body weight. The r values derived for correlations between body weight and protease or amylase activity per gram dry weight of pancreas were not significant so no adjustment was

Table XXII

Effect of Dietary Chlortetracycline on Dry Weight and Hydrolase Activities[1] of the Pancreas

Lot		1[2]	2[2]	Between treatment	Necessary F value	
Chlortetracycline		-	+	F value	0.05	0.01
Dry weight	gm.	11.7	15.2	15.83	5.12	10.56
Adjusted dry weight	gm.	12.5	14.4	3.07	5.32	11.26
Total protease	gm.	28.9	44.3	7.90	5.32	11.26
Adjusted total protease	gm.	29.3	44.0	3.31	5.59	12.25
Total Amylase	gm.	29.3	47.3	49.65	5.12	10.56
Adjusted total amylase	gm.	30.3	46.2	14.38	5.32	11.26
Protease activity/gm. dry weight	gm.	2.3	2.9	3.74	5.32	11.26
Amylase activity/gm. dry weight.	gm.	2.3	3.2	14.74	5.12	10.56

1) Hydrolase activities are listed in terms of the activities of equivalent weights of standards prepared as follows:
Protease - 3 parts Pancreatin 3 X USP plus 1 part Intestinal Hog Mucosa
Amylase - Pancreatin 3 X USP.

2) Means of determinations for 10 pigs with the exception of the data for protease activity which are means of determinations for 9 pigs.

attempted on these data. Mean values obtained together with the results of analysis of variance are summarized in Table XXII.

From Table XXII it may be noted that:

a. the dry weights of the pancreas glands from the pigs fed chlortetracycline were greater than those of their controls, but when adjusted for body weight the differences were not significant.

b. pair-mate differences in the total protease activity of the pancreas were significant at the 5% level before, but not after, adjustment for live weight of the pigs.

c. the corresponding differences for amylase activity were significant at the 1% level both before and after adjustment for body weight.

d. pair-mate differences in amylase activity per gram dry weight of pancreas were significant at the 1% level.

Fat Content of Pancreas

The mean crude fat content of the pancreas glands of 6 pairs of pigs placed on test after the above experiments were completed was 14.65% for the control and 14.60% for the pigs supplemented with chlortetracycline. These results indicate that it is highly improbable that differences existed in the fat content of the pancreas glands from control and antibiotic-fed animals of the first 10 pairs. Furthermore it was found that the fat

content of the pancreas was not significantly correlated with body weight.

Discussion

Reports in the literature do not agree concerning growth response obtainable from antibiotic supplementation of the ration when feed intake is restricted. Experiments with swine reported by Brown and coworkers (21) and with chicks by Scott and coworkers (86) indicate that the growth response to dietary antibiotics is mainly the result of increased feed intake, whereas Davis and Briggs (39) obtained an increased rate of gain in chicks and poults with antibiotic supplements when feed intake was limited to the amounts consumed by the non-supplemented lots. In the present experiments the average daily gain for 3 pigs self-fed the basal ration supplemented with chlortetracycline was 63% greater than that of their controls, while the corresponding mean difference in favor of the supplemented ration was 14% for the 13 pairs fed under the limited paired-feeding technic.

The data in Table XXII show that animals receiving dietary chlortetracycline had larger pancreas glands and greater stores of pancreatic protease and amylase than did their controls. The fact that after adjustment for body weight the differences were not significant for pancreas dry weight or protease activity indicates that

the effects of the ingested antibiotic on rate of gain, pancreas dry weight and pancreatic protease were of similar magnitude.

In the case of amylase, however, the observed differences between test and control pair-mates are not explainable on a basis of increased rate of gain and final size of animal because these differences remained significant after adjustment to a basis of equal body weights. This conclusion is confirmed by data in the last line of Table XXII, which show that the pancreas glands of pigs fed the antibiotic contained significantly more amylase per gram dry matter than did those of their pair-mates. These observations suggest that favorable changes in both the rate of the growth of the pancreas and the rate of amylase production per unit mass of this gland were associated with the feeding of chlortetracycline.

PAPER III - THE EFFECT OF DIETARY CHLORTETRACYCLINE ON THE PROTEASE, AMYLASE AND CELLULASE ACTIVITY OF THE CONTENTS OF THE SMALL INTESTINES AND OF THE CECA OF YOUNG GROWING SWINE

Abstract

Four treatments, involving differences in method of feeding and interval between last feed and slaughter, were employed in a study of the effect of dietary chlortetracycline on the protease, amylase and cellulase activity in the intestinal and cecal contents of 16 pairs of weanling pigs. Most consistent results were obtained with 6 pairs which were limited pair-fed except for the final feeding during which feed was available *ad libitum* for a four-hour period ending 18 hours before slaughter. For these 6 pairs the mean total activity of all three hydrolases in the contents of the small intestines and of the ceca of the antibiotic-fed animals was significantly greater than in those of the control animals. Expressed as activity per gram dry matter of intestinal contents, differences in favor of the pigs receiving chlortetracycline were significant for protease and amylase but not for cellulase. When the combined results obtained from all 16 pairs of the experimental animals were analysed, the results showed that on a basis of activity per gram dry matter of intestinal contents, ingested chlortetracycline induced significant increases in amylase and cellulase

but not in protease activity. Amylase and cellulase activity per gram dry matter of cecal contents were higher for pigs fed the antibiotic than for their controls. The mean wet weight of the empty small intestine and the mean dry weight of the mucosa scraped from the anterior 3-meter section of the small intestines were lower for the chlortetracycline-fed animals but the differences were not statistically significant.

Introduction

A number of reports are available to indicate that ingested antibiotics may, through their effect on cellulolytic rumen micro-organisms, influence cellulose digestion in ruminants. However, to the author's knowledge, the possibility that ingested antibiotics may modify the activity of enzymes present in the lumen of the intestine has not been investigated.

In Paper II evidence was presented to show that dietary chlortetracycline increased protease and amylase content of the pancreas. Enzymes from the pancreas, the intestinal mucosa and microorganisms present in the digestive tract are chiefly responsible for protein and carbohydrate digestion in the small intestine and cecum. The main purpose of the present study was to determine whether differences in protease, amylase or cellulase activity could be demonstrated between

homogenates prepared from intestinal or cecal contents of pigs fed chlortetracycline at a level of 20 gm. per ton of ration, and those prepared from control pigs not fed the antibiotic.

From results obtained with chicks, Coates and coworkers have suggested (31, 32) the possibility that, as a result of a lower level of infection in the intestines of animals fed antibiotics, the intestinal wall may be thinner and thus presumably absorption may be more efficient than in animals not fed antibiotics. In this connection a minor objective of the work reported here was to determine whether differences might be demonstrable between control pigs and those fed chlortetracycline with respect to (a) wet weight of the small intestine, and (b) dry weight of scrapings from the mucosa of the anterior 3 meters of the small intestine.

Experimental

Materials and Methods

Material for the assays discussed below was collected from the 16 pairs of pigs used for the study of the effects of dietary chlortetracycline on the pancreas as described in Paper II. The small intestine and cecum were excised and brought to a room kept at 5°C. where the contents were expelled, a sample taken for a dry

matter determination and the remainder stored for 18 to 20 hours before homogenization at 5°C. in electric blenders. Homogenates were prepared by the procedure described for pancreatic homogenates at a concentration of 1 gm. of wet intestinal or cecal contents in 5 ml. of distilled water. Prior to assay homogenates were stored under toluene at 1°C. for 18 hours in the case of protease and amylase, and 2 hours in the case of cellulase. Protease, amylase and cellulase assays were conducted on appropriate dilutions by the methods described in Paper I.

In connection with the viscosimetric method of cellulase assay employed it was found that the relative viscosities of mixtures of 1 ml. of dilutions of homogenates of intestinal or cecal contents and 9 ml. of water were the same after as before incubation for 18 hours at 37°C. It was also found that after tubes containing 8 ml. carboxymethyl cellulose and 1 ml. of Enzyme 19 extract were incubated for 18 hours, the addition of 1 ml. of fresh homogenate of a given concentration produced the same increase in flow time through Ostwald viscosimeters as did the addition of 1 ml. of the same homogenate to 9 ml. of water. From these observations it was concluded that in the assay method, correction for the increase in viscosity attributable to the homogenate *per se*

could be made by calculating relative viscosity as follows:

Flow time of 5 ml. aliquot of an 18-hour hydrolysate of 8 ml. carboxymethyl cellulose solution + 1 ml. homogenate dilution of concentration X + 1 ml. water ÷ flow time of 5 ml. aliquot of freshly prepared mixture of 9 ml. water + 1 ml. homogenate of concentration X.

Recovery Trials

In preliminary trials known concentrations of reference protease, amylase and cellulase preparations as described in Paper I were added to one-half of the contents of the small intestines and of the ceca of 2 pigs prior to homogenization, while the other half was homogenized without the addition of any enzyme. The results of assays conducted on these homogenates indicated recoveries of added hydrolases as follows: (1) from intestinal material - 96% for protease and amylase, and 97% for cellulase; (2) from cecal material - 101% for protease and amylase, and 96% for cellulase.

Preslaughter Treatment of Experimental Pigs

The selection of a satisfactory time interval after the ingestion of feed at which to kill the experimental animals and collect intestinal and cecal contents for assay presents some problems. Studies of gastro-intestinal

movements in young swine by Neimeir, as summarized by Dukes (42) indicated that for about one hour after feeding, ingesta were passed relatively rapidly from the stomach to the duodenum to the jejunem. The jejunem was usually empty within 8 to 12 hours of feeding. Ileal motility diminished about 5 hours and cecal motility between 6 and 8 hours after feed was offered. Balls of feces began to leave the colon 14 to 16 hours after feed was given.

It may be concluded from existing knowledge of mechanisms controlling rate of secretion of hydrolases active in the intestine that the rate of secretion of these enzymes would be highest during periods shortly after food is eaten and during which ingesta are moving rapidly through the upper regions of the small intestine. Slaughter of the experimental animals at this time might thus be expected to permit the collection of material from the small intestine with a higher total enzyme activity, but not necessarily a higher activity per unit dry matter than might be observed in intestinal contents from animals slaughtered some hours after they had eaten. It is even more difficult to theorize regarding the selection of an interval between final feeding and slaughter which might be expected to allow for the collection of cecal contents for which assay results

would be of most significance in relation to digestion in the live animal. For these reasons, and others discussed below, the intestinal and cecal contents assayed in this study were collected from variable numbers of pairs of pigs which had been subjected to four different treatments.

Treatment A

Three pairs of weanling pigs fed *ad libitum* were starved for 22 - 23 hours before being slaughtered by pairs after they had been on test for 2, 4 or 6 weeks. Under this treatment the pigs fed chlortetracycline were about 30% heavier at slaughter than their pair-mates, while the dry weights of the contents of their intestines and ceca were approximately twice as great. Since it was obvious that both these factors would complicate interpretation of enzyme activity assays the practice of self-feeding was abandoned.

Treatment B

The 2 pairs of weanling pigs in this treatment were hand-fed, the daily feed allowance for pair-mates being limited to the amount consumed the previous day by the mate consuming the least. At 22 - 23 hours before slaughter after 2 or 4 weeks on test each animal of a pair was offered a final feed in the amount of 1/2 that

consumed the previous day. It was anticipated that by this procedure it might be possible to reduce the differences between pair-mates observed in Treatment A with respect to live weight and weights of intestinal and cecal contents.

Within pair differences in body weight were reduced considerably, but the mean dry weight of intestinal contents of the control pigs was 67% greater than that of their mates while the dry weight of the cecal contents from the pigs fed chlortetracycline was 52% more than that of their controls. It was concluded that these differences were attributable to the fact that although both members of a pair were offered their final feed at the same time, the pigs receiving the antibiotic had eaten all their feed at least 2 hours before the control animals had finished theirs.

Treatment C

Five pairs of 8 to 9 week old pigs, fed by the limited paired feeding technic described above, were killed at 2, 4, 6 (2 pairs) or 8 weeks, after being offered 1/2 the weight of feed consumed the previous day at 22 - 23 hours, plus approximately 1/6 of this amount at 2 hours, before slaughter. Under this treatment extreme variability was observed within and between pairs

in weight of intestinal contents, and in the total as well as in the per gram dry weight, protease, amylase and cellulase activities of this material. Collection of assay material from pigs slaughtered during a highly active stage of digestion was therefore discontinued.

Treatment D

The 6 pairs of weanling pigs in this group were limited pair fed except for the final feeding when they were fed *ad libitum* for 4 hours, the feed being removed 18 hours before slaughter. In order to minimize differences in body weight between animals slaughtered at different times, 3 pairs were killed at 3 weeks and 3 pairs at 4 weeks after they were put on test. Under this treatment the average dry weights of the intestinal contents of control and antibiotic-fed pigs were equal, while, presumably due to the fact that they ate more at the final *ad libitum* feeding, the average weight of cecal contents was 36% higher for the pigs fed chlortetracycline than for the controls. The mean difference in live weight at slaughter was only 1 lb. in favor of the pigs fed the antibiotic. The variability in hydrolase assay results for samples collected from the small intestine was much lower than that for samples obtained under treatment C.

Results

Contents of Small Intestine

In Table XXIII the mean results are shown for the 6 pairs of pigs in treatment D and for all 16 pairs in treatments A, B, C and D. In both cases the data were analyzed by the paired t-test. The results for treatment D indicate that, expressed as total activity or as activity per gram dry weight, more protease and amylase were present in the intestinal contents of pigs fed chlortetracycline than in those of their controls. Although the total cellulase activity of the intestinal contents of the test animals was significantly greater than that of the controls, this difference was not significant when calculated to a per unit dry matter basis.

It is of interest to note that despite the great variability of the assay results obtained on intestinal contents collected under different treatments, the results of the analysis on the combined data indicate the existence of higher levels of amylase and cellulase per unit dry matter of intestinal contents from the pigs fed the antibiotic. For all 16 pairs of pigs the mean protease activity per gram dry matter of intestinal contents was approximately 10% higher for the pigs fed the antibiotic than for the controls, but this difference

did not prove to be significant.

The observed degree of increase in hydrolase activity per gram dry matter of contents of the small intestine associated with the feeding of chlortetracycline was approximately twice as great for amylase as for protease. A paired t-test on the combined results for all animals expressed as a ratio, protease activity per gm. dry weight:amylase activity per gm. dry weight, indicated significance for the probability that the effect of dietary chlortetracycline on amylase activity was more favorable than on protease activity in the small intestine.

Contents of the Cecum

The mean dry weights, hydrolase activities and results of paired t-test analyses on data for cecal material are summarized in Table XXIV. Regardless of the pre-slaughter treatment, the weight and total hydrolase activities of cecal contents of animals fed chlortetracycline were greater than those of their controls. Possible explanations for the greater weight of cecal material in pigs fed the antibiotic have been mentioned in the descriptions of the 4 pre-slaughter treatments employed.

On a per gram dry matter basis, increases in enzyme

Table XXIII

Effects of Dietary Chlortetracycline on Hydrolase Activities[1] in Contents of the Small Intestine

Treatment		D			A B C D	
Lot	1	2	2-1	1	2	2-1
Chlortetracycline	-	+		-	+	
No. pigs	6	6		16	16	
Mean dry wt. intestinal contents gm.	19.6	19.4	-0.2	36.5	35.3	-1.2
Mean total protease . . . . gm.	7.6	9.7	2.1*	20.3	18.8	-1.5
Mean protease/gm. dry wt. . . . . mg.	401	504	103*	456	503	47
Mean total amylase . . . . gm.	0.9	1.5	0.6***	2.1	2.3	0.2
Mean amylase/gm. dry wt. . . . . mg.	51	77	26**	53	66	13*
Mean total cellulase . . . . mg.	3.1	4.8	1.7**	7.4	10.2	2.8*
Mean cellulase/gm. dry wt. . . . mg.	0.2	0.3	0.1	0.2	0.3	0.1**
Protease/gm. dry wt. / Amylase/gm. dry wt.	8.9	6.5	-2.4	9.9	7.5	-2.4*

(1) Hydrolase activities are listed in terms of the activities of equivalent weights of standards prepared as follows:
Protease - 3 parts Pancreatin 3X USP plus 1 part Intestinal Hog Mucosa
Amylase - Pancreatin 3X USP
Cellulase - Enzyme 19.

* $P < 0.1$
** $P < 0.05$
*** $P < 0.01$.

Table XXIV

Effects of Dietary Chlortetracycline on Hydrolase Activities(1)
in Contents of the Cecum

Treatment	D			A B C D		
Lot	1	2	2-1	1	2	2-1
Chlortetracycline	-	+		-	+	
No. pigs	6	6		16	16	
Mean dry wt. cecal contents . . gm.	26.6	36.2	9.5	22.3	29.6	7.7***
Mean total protease . . . . mg.	368	661	293**	309	544	235***
Mean protease/gm. dry wt. . . . mg.	13.9	19.0	5.1	15.1	20.0	4.9**
Mean total amylase . . . . mg.	49	136	87**	43	121	78***
Mean amylase/gm. dry wt. . . . . mg.	1.9	3.8	1.9**	2.1	4.6	2.5**
Mean total cellulase . . . . mg.	44	105	61**	37	88	51***
Mean cellulase/gm. dry wt. . . . mg.	1.9	2.9	1.0**	1.8	2.9	1.1***

(1) As (1) Table XXIII.

** $P < 0.05$
*** $P < 0.01$.

activity were significant for protease, amylase and cellulase when the results for all 16 pairs were combined. When the data for samples collected under treatments C and D were analyzed separately, increases in protease and cellulase activity per gram dry matter were found to be significant at the 10% level in the case of treatment C, while for treatment D corresponding increases in amylase and cellulase activity were significant at the 5% point. Protease and amylase activities per gram dry matter of cecal contents were much smaller than those observed for contents of the small intestine, whereas the cellulase activity of cecal contents was much greater than that of intestinal contents.

Weights of Small Intestines and of Mucosa from the Anterior Three Meters

After adhering tissue had been excised from the exterior wall of the intestine and the contents expelled by light pressure between the thumb and forefinger, the intestine was cut into 3 to 4 foot lengths and the lumen flushed carefully with distilled water to remove remaining particles of ingesta. The combined weight of the sections

was taken after they had been hung up to drain for two hours at 5°C. The anterior 3 meter section was everted, blotted dry and scraped with the edge of a microscope slide to remove the mucosa. Dry matter determinations were made by drying samples to a constant weight in a vacuum oven at 95° - 100°C. Results obtained are summarized in Table XXV.

Table XXV

Effects of Dietary Chlortetracycline on Wet Weight of Small Intestine and on Dry Weight of Mucosa

Lot Chlortetracycline	1 -	2 +	Between treatment F value
Wet wt. small intestine[1)]. gm.	1028	996	0.38
Adj. wet wt. small intestine . . . . gm.	1060	964	0.77
Dry wt. intest. mucosa[2)] . gm.	9.9	9.0	0.78
Adj. dry wt. intest. mucosa . . . . gm.	10.4	8.4	0.76

1) Means for 16 pairs.

2) Means for 10 pairs from treatments A, B and C.

Results

Analyses of the data indicated the existence of a highly significant correlation (r = 0.68) between body weight and wet weight of small intestine, and of a significant correlation (r = 0.55) between body weight

and dry weight of mucosa from the anterior 3 meters of the intestine. The results obtained for these characteristics were therefore adjusted for body weight by analysis of covariance. On the adjusted basis the mean wet weight of the intestine was approximately 10% and the mean dry weight of mucosa approximately 20% less for the pigs fed the antibiotic as compared to the controls.

Discussion

Data indicating increased content of protease and amylase of the pancreas glands of pigs fed chlortetracycline at a level of 20 gm. per ton of ration were presented in Paper II. As reported above dietary chlortetracycline was also associated with higher levels of protease and amylase in the contents of the small intestine and of the cecum. Increased production by the pancreas might account for the higher levels of these enzymes observed in the intestinal and cecal contents of the antibiotic-fed pigs.

The fact that homogenates of intestinal and especially of cecal material from pigs fed chlortetracycline exhibited higher cellulase activity than did those from their controls, suggests the interesting possibility that, contrary to what might be expected from the results of most *in vitro* studies regarding the effect of antibiotics

on cellulolytic activity of rumen microorganisms, dietary chlortetracycline may stimulate the production of cellulases by microorganisms in the digestive tract of swine. However, other hypotheses may be advanced to explain the observed increases in cellulase activity at least of cecal contents of the pigs fed chlortetracycline. For example, if more complete digestion and absorption of starch and protein are assumed, increased cellulase production could arise from an adaption of cellulolytic microorganisms to a resulting higher concentration of cellulose.

A number of workers (16, 79, 99) have reported small increases in carcass fat associated with the feeding of antibiotics to swine. Since the deposition of fat in this species normally depends largely on the digestion, absorption and metabolism of starchy feed, it is possible that a tendency toward increased carcass fat in antibiotic-fed hogs may be associated with an especially favourable effect of antibiotics on amylase levels in the digestive tract.

It is recognized that data from the limited number of *in vitro* studies presented in Papers II and III probably do not provide an accurate reflection of digestive processes in the intact animal. Nonetheless they do furnish some evidence in favour of the hypothesis that part of the favourable effect of dietary antibiotics

as measured by rate of gain may be due to a favorable effect on digestive enzyme production.

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